ELSEVIER

FEBS Letters 342 (1994) 230–234

FEBS 13860

FEBS LETTERS

The accessibility of peptides bound to the mouse MHC class II molecule IE^d studied by fluorescence

Anton I.P.M. de Kroon*

Department of Chemistry, Stanford University, Stanford, CA 94305, USA

Received 17 December 1993; revised version received 25 February 1994

Abstract

The accessibility of fluorescently labeled (antigenic) peptides bound to the detergent-solubilized mouse MHC class II protein IE^d has been studied by fluorescence techniques. Based on the efficiency of fluorescence quenching by the aqueous quenchers iodide and TEMPOL, different degrees of accessibility of the peptide-attached fluorescein moiety are distinguished in the IE^d-bound state, which depend on the nature of the peptide and on the site of attachment. These different extents of sequestration from the aqueous phase are reflected in the fluorescence properties of the corresponding NBD-labeled peptides bound to IE^d. The results provide information on the topology of class II bound peptides.

Key words: MHC class II; Fluorescence quenching; Fluorescein-labeled peptide; NBD-labeled peptide

1. Introduction

MHC class II proteins are heterodimeric polymorphic membrane proteins that bind antigenic peptides and present them to T cells. Recently, the three-dimensional structure of the human class II protein HLA-DR1 was solved by X-ray crystallography [1]. As predicted from sequence homology [2], the class II peptide binding site is very similar to that of the MHC class I molecule of which the crystal structure was determined earlier [3]. Both consist of a groove between two parallel α-helices which are on top of a continuous eight-strand antiparallel β-sheet. One important difference between the class I and class II binding sites is that the ends of the class II groove are more open, allowing the peptides, which are bound in an extended conformation, to protrude from both ends [1]. This is consistent with the ability of class II proteins to bind peptides of variable length [4,5]. In contrast, class I proteins bind peptides of a limited length of 8–10 amino acids [6], with both the N- and the C-terminus of these peptides anchored in the binding groove and the amino acids in the middle bulging out [7,8]. Apart from X-ray crystallography, 2D NMR techniques have recently been used to characterize the interactions and localization of a peptide in the MHC binding groove [9].

Fluorescent labeling of peptides not only enables the highly sensitive detection of peptide binding to MHC molecules [10], but can also be applied to study the topology of MHC bound peptides, as will be demonstrated here for peptides complexed to the mouse class II molecule IE^d. Using two independent fluorescence methods, (i) quenching of fluorescein emission by aqueous quenchers, and (ii) fluorescence enhancement of NBD-labeled peptides, it is shown that the peptide-attached labels attain different micro-environments upon binding to IE^d. The characterization of the binding to and release from IE^d of the peptides used in this study has been reported [11].

2. Materials and methods

2.1. Purification of IE^d

IE^d was purified from a Nonidet P-40 lysate of A20–1.11 cells by affinity chromatography using the antibody 14.4.4S according to standard procedures [11]. The detergent NP-40 was replaced by *n*-dodecyl β-D-maltoside (DM, Sigma, St. Louis, MO) during the washing of the affinity column. Subsequently, IE^d was eluted using 0.5 M NaCl, 1 mM DM, 0.1 M Na_2CO_3 pH 11.5. The eluate was immediately neutralized to pH 7, concentrated, dialyzed against 150 mM NaCl, 1 mM DM, 10 mM NaP_i pH 7.0 (DM buffer), and stored at 4 °C. The protein concentration was determined by the Lowry method.

2.2. Synthesis and labeling of the peptides

The synthesis, HPLC purification, labeling, and characterization of the fluoresceinated peptides (F-peptides) used in this study has been described [11]. The peptides hen egg lysozyme 107–116 (HEL, H-AWV-

*Corresponding author.

Present address: Centre for Biomembranes and Lipid Enzymology, Department of Biochemistry of Membranes, Padualaan 8, 3584 CH Utrecht, The Netherlands. Fax: (31) (30) 522 478.

Abbreviations: MHC, major histocompatibility complex; HEL, hen egg lysozyme 107–116; dyn, dynorphin A 1–13; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy; F, fluorescein; NBD, 7-nitro-benz-2-oxa-1,3-diazole; ACP, acyl carrier protein 65–74.

SSDI 0014-5793(94)00245-Q

AWRNRCK-OH) and dynorphin A 1–13 (dyn, H-YGGFLRRIRPKLK-OH) were labeled with NBD at the N-terminus while still on the resin, by reaction with a 3-fold molar excess of 4-fluoro-7-nitrobenz-2-oxa-1,3-diazole (NBD-fluoride, Molecular Probes, Eugene, OR) in dimethylformamide to yield NBDHEL and NBDdyn, respectively. The cysteine residue of NBDHEL was carboxamidomethylated with iodoacetamide to prevent the formation of peptide dimers. Alternatively, the cysteine residue at position 115 of HEL 107–116 was labeled with NBD by reaction with a 3-fold molar excess of *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)amino-7-nitrobenz -2- oxa-1,3 diazole (IANBD, Mol. Probes) at 1 mg peptide/ml in 0.1 M ammonium-acetate pH 8.0 containing 25% v/v acetonitrile, to yield NBDcysHEL.

The final purity of the peptides was greater than 98%. Peptide stock solutions in water were stored in aliquots at −20 °C. The concentrations of the peptide stock solutions were determined by quantitative amino acid analysis and confirmed by spectrophotometry as described [11]. Both NBDHEL and NBDdyn in PBS exhibited an ε^{476} of $2.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$, and ε^{494} of NBDcysHEL was found to be $2.8 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ in agreement with values reported in the literature [12,13].

2.3. Preparation of IE^d–peptide complexes

The class II molecules (1–3 μM) were incubated with 50 μM of the fluorescein (F)- or NBD-labeled peptides in DM buffer at 37 °C for 5 days. Complexes were separated from the bulk of free peptide by gel filtration on Sephadex G-50. For the final purification, the eluate was concentrated and applied to a TSK-gel G3000 SW (Tosohaas, Montgomeryville, PA) high-performance size exclusion column (HPSEC) eluted with DM buffer [11]. The IE^d $\alpha\beta$ heterodimer fraction was collected and used within a few hours in the fluorescence measurements. The concentration of the complex was determined from the absorbance signal of the UV detector connected to the column.

2.4. Fluorescence measurements

All fluorescence measurements were carried out at room temperature, in DM buffer, in a total volume of 1 ml, and under continuous stirring, using a Spex Fluorolog fluorimeter. In the fluorescence quenching experiments the fluorescence intensity of the IE^d–Fpeptide complex (conc. 7–10 nM) and of free F-peptide with a corresponding fluorescence level (0.5–1 nM) was recorded after the successive addition of small aliquots of quencher stock solutions. The excitation wavelength was 490 nm, the emission was read at the peptide's wavelength of maximum emission, which is 526 nm for FHEL and 516 nm for the other F-peptides studied. The fluorescence intensity of the complex was corrected for background, i.e. the fluorescence intensity of the eluate collected from the HPSEC column prior to the IE^d fraction, which never exceeded 5% of the total signal. The quencher stock solution was either 4 M KI containing 1 mM $Na_2S_2O_3$ to avoid I_3^- formation, or 0.4 M 4-hydroxy-2,2,6,6-tetramethylpiperidinyloxy (TEMPOL, Aldrich Chemical Co., Milwaukee, WI). The quenching data were analyzed according to the Stern–Volmer equation, $F_o/F = 1 + K_{SV} \cdot [Q]$, with F_o the fluorescence intensity in the absence of quencher Q, F the fluorescence measured at different values of [Q], and K_{SV} the Stern–Volmer constant which provides a relative measure for the fluorophor's accessibility to the quencher.

3. Results and discussion

The antigenic peptide HEL 107–116 was labeled with fluorescein either at its N-terminus or at Cys^{115}, yielding FHEL and FcysHEL, respectively. The modification of the Cys residue abrogates the IE^d-restricted activation of the HEL 107–116 specific T cell hybridoma D2.1E5, whereas labeling of the N-terminus does not interfere with T cell activation [11,14]. Both fluoresceinated peptides show a similar extent of binding to detergent-solubilized IE^d, which results in IE^d-peptide complexes with half times of dissociation at pH7, 37°C of 100 h and 200 h for FcysHEL and FHEL, respectively [11]. Taking advantage of the high stability of these complexes, fluorescence quenching experiments were performed on IE^d-Fpeptide complexes purified by HPSEC. Under the experimental conditions used, class II-peptide complex preparations with approximately 10% of the class II molecules occupied by the fluoresceinated peptide were obtained (not shown). The efficiency of quenching by aqueous quenchers was compared for the IE^d-bound F-peptide and a corresponding amount of the free F-peptide in DM buffer.

The accessibility of the fluorescein moiety to the collisional quencher of fluorescein fluorescence iodide [15], is reduced when the peptides FHEL and FcysHEL are bound to IE^d as shown in the Stern–Volmer plot (Fig. 1A), with the reduction in I^- accessibility of FHEL being greater than that of FcysHEL. As the putative binding

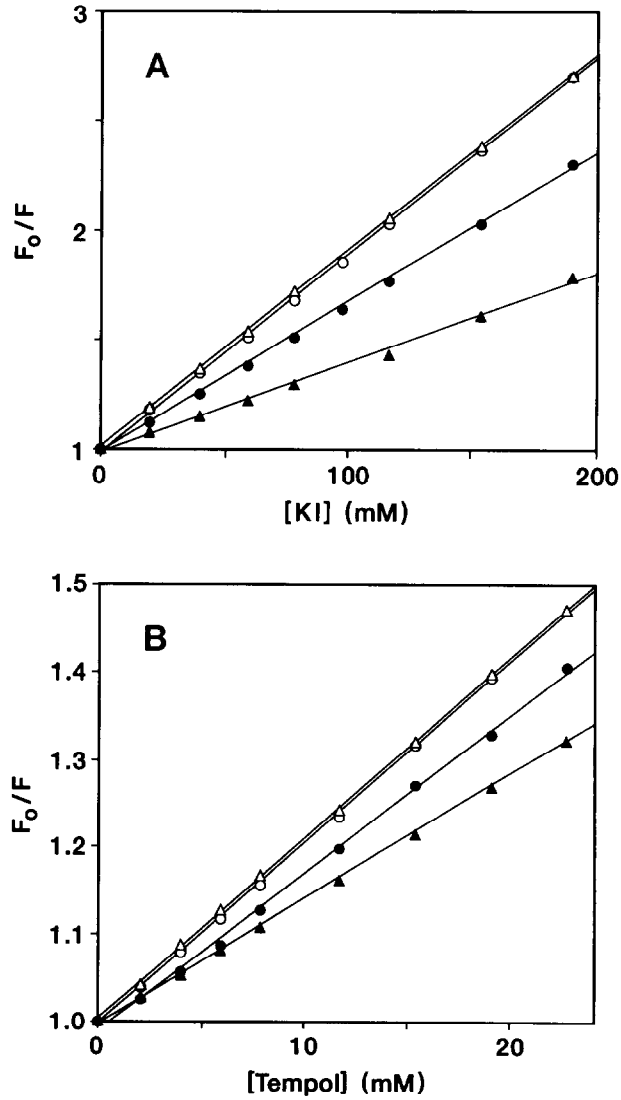

Fig. 1. Stern–Volmer plots of quenching by iodide (A) and TEMPOL (B) of FHEL (triangles) and FcysHEL (circles) bound to IE^d (solid symbols) and in DM-buffer (open symbols). The concentration of the IE^d-peptide complexes is in the range of 7–10 nM, that of the free peptides in the range of 0.5–1 nM. For experimental details see section 2.

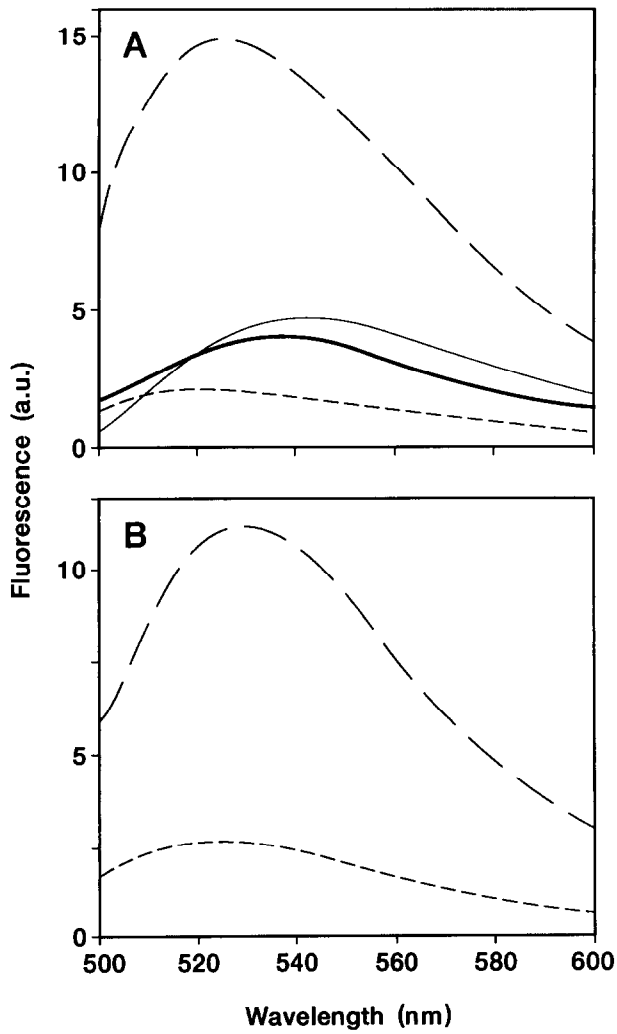

Fig. 2. (A) Fluorescence emission spectra of NBDHEL in DM-buffer (short dashes) and bound to IE^d (long dashes), of NBDcysHEL in DM-buffer (thin line) and bound to IE^d (thick line), and of (B) NBDdyn in DM-buffer (short dashes) and bound to IE^d (long dashes). The concentration of the IE^d-peptide complexes is 40 nM. In view of the poor signal-to-noise ratio, the spectra of the complexes were smoothed. Spectra of the free peptides in DM-buffer were recorded at 50 nM and depicted after normalization to 4 nM to enable comparison with the spectra of the IE^d-bound peptides, which are estimated to occupy ~ 10% of the IE^d molecules (see text), assuming that the binding of NBD- and fluorescein-labeled peptides is similar.

groove of IE^d is lined with negatively charged amino acid residues [16], this reduced quenching may be due to electrostatic repulsion of the anionic I^- [17]. Therefore the experiment was repeated using the uncharged nitroxide radical TEMPOL, which quenches fluorescein fluorescence very efficiently by a mechanism involving electron exchange [18]. Although the decrease in TEMPOL quenching efficiency relative to the free peptide is smaller, the decreased accessibility of the IE^d-bound peptides to TEMPOL parallels that to I^- (Fig. 1).

In Table I the values of the Stern–Volmer constants K_{SV} of several peptides in the IE^d-bound state and free in solution have been summarized with the residual accessibility to the quenchers calculated as the ratio of $K_{SV}^{bound}/K_{SV}^{free}$. The disulfide-bridged dimer of HEL 107–116 was previously shown to compete for binding to IE^d with F(cys)HEL, and to displace prebound peptides from IE^d. Complexes of its fluoresceinated analogue $FHEL^{S\text{-}S}HEL$ with IE^d have intermediate stability compared to the FcysHEL- and FHEL complexes [11]. Quenching of this complex with TEMPOL reveals that the residual accessibility of the fluorophor in the IE^d-bound state is virtually the same as that in the FHEL-IE^d complex (Table 1). Under the reasonable assumption that one of the HEL 107–116 sequences in the dimer uses the same anchoring site(s) in the binding groove as the monomer [11], this result implies that there is a preference for the fluoresceinated N-terminus over the open one to take this position.

The fluorescein attached to the N-terminus of the peptide dynorphin A 1–13 (Fdyn) also shows strong sequestration from the aqueous phase upon binding to IE^d (Table 1). Although not known as an IE^d-restricted antigenic peptide, dynorphin was shown to specifically bind to IE^d resulting in a stable complex ($t_{1/2}$ ~ 50 h at 37°C), and to be very effective in displacing peptides from preformed IE^d-peptide complexes [11,19,20].

As a control the quenching of the randomly chosen peptide FACP was examined. The unlabeled analogue ACP (acyl carrier protein 65–74: H-VQAAIDYING-OH) does not compete for binding to IE^d with F(cys)HEL, neither does unlabeled HEL compete with FACP [11]. The low level but long-lived ($t_{1/2}$ ~ 100 h at 37 °C) association of FACP with IE^d detected with HPSEC was therefore attributed to non-specific binding outside the binding groove. Accordingly, no significant difference in accessibility to TEMPOL was detected for FACP free in solution or associated with IE^d (Table 1).

Besides fluorescein-labeled peptides, also peptides labeled with NBD have been used to detect peptide binding

Table 1
Stern–Volmer constants of quenching (K_{SV}) by iodide and TEMPOL of the fluoresceinated peptides bound to IE^d, free in DM-buffer, and their ratio

Peptide	Iodide			TEMPOL		
	K_{SV}^{bound} (M^{-1})	K_{SV}^{free} (M^{-1})	$K_{SV}^{bound}/K_{SV}^{free}$	K_{SV}^{bound} (M^{-1})	K_{SV}^{free} (M^{-1})	$K_{SV}^{bound}/K_{SV}^{free}$
FHEL	3.6	8.9	0.40	14.3	20.5	0.70
FcysHEL	6.9	9.1	0.76	18.1	20.7	0.87
$FHEL^{S\text{-}S}$ HEL	–	–	–	13.6	19.5	0.70
Fdyn	2.8	8.5	0.33	15.9	24.0	0.66
FACP[1]	–	–	–	15.4	15.9	0.97

[1]In view of the low level of association of the control peptide FACP with IE^d, the concentration of complex used in this experiment was 40 nM.

to MHC molecules [21]. In contrast to fluorescein, the fluorescence characteristics of NBD are dependent on the polarity of the surrounding medium [12]. As the environment becomes more apolar (smaller dielectric constant) the emission quantum yield of NBD increases while the wavelength of maximum emission decreases. Using the NBD-labeled analogues of HEL 107–116 and dynorphin, the effects of binding to IE^d on the NBD emission spectra were examined. In Fig. 2 the emission spectra of NBDHEL, NBDcysHEL, and NBDdyn complexed to IE^d are compared to those of the corresponding amounts of the free peptides in DM buffer. The wavelength of maximum emission and the quantum yield of NBDcysHEL are higher than those of both N-terminally labeled peptides which is probably due to the different chemistry by which the fluorophores are coupled.

Upon binding to IE^d, both N-terminally labeled peptides exhibit a strong enhancement in fluorescence intensity, indicating that the NBD label has entered a less polar environment. In contrast, the fluorescence intensity of NBDcysHEL does not significantly change, indicating that the NBD label stays in a polar environment when bound to IE^d. The weak fluorescence signal from the complexes does not allow conclusions about possible shifts in emission wavelength (see legend to Fig. 2).

The results obtained using the NBD-peptides are consistent with the quenching data. Although the cysteine-attached fluorophore of HEL 107–116 becomes less accessible to the aqueous quenchers upon binding to IE^d, the data obtained with NBDcysHEL indicate that the label at this position does not enter a more apolar environment. Probably the interaction of the peptide with the IE^d binding groove results in some extent of steric shielding of the fluorophor from the quenchers while leaving the local dielectric constant experienced by the fluorophor unaffected. Taking into account the inability of both FcysHEL [11] and NBDcysHEL (not shown) to elicit IL-2 production by an HEL 107–116 specific T cell hybridoma, it is concluded that the cysteine-attached label of HEL 107–116 bound to IE^d is exposed to the aqueous phase.

The analysis of HEL 107–116 analogues with single amino acid substitutions has revealed that the three positively charged residues at positions 112, 114, and 116 determine the peptide's capacity to bind to IE^d [19]. The results of the present study indicate that the fluorescent label attached at the opposite end of the molecule, at the N-terminus, becomes at least partially sequestered from the aqueous phase. Interestingly, the nature of the residue at this position is not important for the interaction with IE^d [19]. The N-terminus of dynorphin appears to attain a similar localization. The data point to a structural model in which the N-termini of HEL and dyn are buried in, rather than protruding from, the IE^d binding groove. In this respect the IE^d–peptide complexes studied here, differ from the complex of IE^k with the moth cytochrome *c* 88–103 peptide where the N-terminus was shown to extend out of the binding groove [9,17]. Given the ability of the class II binding groove to accomodate a stretch of approximately 15 amino acid residues [1], the shorter lengths of HEL and dyn compared to the cytochrome *c* peptide may account for this difference.

In a previous study in which an anti-fluorescein antibody was used as quencher, the authors were not able to detect any differences in accessibility of fluorescein labels attached at various positions, including the N-terminus, of the peptide OVA 323–339 bound to IA^d [22]. The discrepancy between these results and the data presented here may be due to the different nature of the quenchers used. TEMPOL and iodide do not irreversibly interact with fluorescein like the antibody and therefore report more reliably on the time-averaged exposure of the fluorescein probes to the aqueous phase.

In conclusion, the straight-forward technique of fluorescence quenching of fluoresceinated peptides complexed to MHC molecules, which requires only small amounts of peptide and protein, provides information on the structure of these complexes that may complement the more elaborate crystallographic and 2D NMR studies.

Acknowledgements: The author wishes to thank Dr. Harden M. McConnell for helpful discussions. This work was supported by a fellowship from the Netherlands Organization of Scientific Research (NWO).

References

[1] Brown, J.H., Jardetzky, T.S., Gorga, J.C., Stern, L.J., Urban, R.G., Strominger, J.L. and Wiley, D.C. (1993) Nature 364, 33–39.
[2] Brown, J.H., Jardetzky, T., Saper, M.A., Samraoui, B., Bjorkman, P.J. and Wiley, D.C. (1988) Nature 332, 845–850.
[3] Bjorkman, P.J., Saper, M.A., Samraoui, B., Bennett, W.S., Strominger, J.L. and Wiley, D.C. (1987) Nature 329, 506–518.
[4] Rudensky, A.Y., Preston-Hurlburt, P., Hong, S.C., Barlow, A. and Janeway Jr., C.A. (1991) Nature 353, 622–627.
[5] Hunt, D.F., Michel, H., Dickinson, T.A., Shabanowitz, J., Cox, A.L., Sakaguchi, K., Appella, E., Grey, H.M. and Sette, A. (1992) Science 256, 1817–1820.
[6] Falk, K., Rotzschke, O., Stevanovic, S., Jung, G. and Rammensee, H.G. (1991) Nature 351, 290–296.
[7] Fremont, D.H., Matsumura, M., Stura, E.A., Peterson, P.A. and Wilson, I.A. (1992) Nature 257, 919–927.
[8] Guo, H.C., Jardetzky, T.S., Garrett, T.P.J., Lane, W.S., Strominger, J.L. and Wiley, D.C. (1992) Nature 360, 364–366.
[9] Driscoll, P.C., Altman, J.D., Boniface, J.J., Sakaguchi, K., Reay, P.A., Omichinski, J.G., Appella, E. and Davis, M.M. (1993) J. Mol. Biol. 232, 342–350.
[10] Tampé, R. and McConnell, H.M. (1991) Proc. Natl. Acad. Sci. USA 88, 4661–4665.
[11] De Kroon, A.I.P.M. and McConnell, H.M. (1994) J. Immunol. 152, 609–619.
[12] Lancet, D. and Pecht, I. (1977) Biochemistry 16, 5150–5157.
[13] Frey, S. and Tamm, L.K. (1990) Biochem. J. 272, 713–719.
[14] Adorini, L., Sette, A., Buus, S., Grey, H.M., Darsley, M., Lehmann, P.V., Doria, G., Nagy, Z.A. and Appella, E. (1988) Proc. Natl. Acad. Sci. USA 85, 5181–5185.

[15] Watt, R.M. and Voss, E.W.Jr. (1979) J. Biol. Chem. 254, 1684–1690.
[16] Bogen, B. and Lambris, J.D. (1989) EMBO J. 8, 1947–1952.
[17] Boniface, J.J., Allbritton, N.L., Reay, P.A., Kantor, R.M., Stryer, L. and Davis, M.M. (1993) Biochemistry 32, 11761–11768.
[18] Matko, J., Ohki, K. and Edidin, M. (1992) Biochemistry 31, 703–711.
[19] Sette, A., Adorini, L., Appella, E., Colon, S.M., Miles, C., Tanaka, S., Ehrhardt, C., Doria, G., Nagy, Z.A., Buus, S. and Grey, H.M. (1989) J. Immunol. 143, 3289–3294.
[20] Pedrazzini, T., Sette, A., Albertson, M. and Grey, H.M. (1991) J. Immunol. 146, 3496–3501.
[21] Babbitt, B.P., Allen, P.M., Matsueda, G., Haber, E. and Unanue, E.R. (1985) Nature 317, 359–361.
[22] Gariépy, J. and Watts, T.H. (1991) Int. Immunol. 3, 279–284.